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Reduced number of interleukin-12 secreting cells in patients with Lyme borreliosis previously exposed to *Anaplasma phagocytophilum*

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Summary

Lyme borreliosis and human granulocytic ehrlichiosis are tick-borne diseases caused by *Borrelia burgdorferi and Anaplasma phagocytophilum*, respectively. Infection with *A. phagocytophilum* has been observed to induce immunosuppression and animal studies suggest that the bacteria might also have prolonged inhibitory effects on immune cells. The aim of this study was to investigate the cytokine secretion in patients exposed previously to *A. phagocytophilum* and currently infected with *B. burgdorferi* compared with patients infected with *B. burgdorferi* and seronegative for *A. phagocytophilum*. Eight patients with erythema migrans and antibodies against *A. phagocytophilum*, 15 patients with erythema migrans and negative *A. phagocytophilum* serology and 15 non-exposed healthy individuals were included in the study. Blood mononuclear cells were stimulated with *Borrelia*-antigen and the number of cytokine [interleukin (IL)-4, IL-5, IL-12, IL-13 and interferon (IFN)-γ]-secreting cells was detected by enzyme-linked immunospot (ELISPOT). This study shows that patients with a previous exposure to *A. phagocytophilum* and a current infection with *B. burgdorferi* have a lower number of *Borrelia*-specific cells secreting IL-12 compared to *Ap* seronegative patients infected with *B. burgdorferi* ($P < 0{\cdot}001$), indicating impairment in the ability to mount strong Th1-responses. We suggest that this mirrors a reduced Th1 response caused by *A. phagocytophilum* which could influence the outcome of the *Borrelia* infection and, speculatively, may also have implications in other conditions.

Keywords: HGE, human granulocytic ehrlichiosis, interleukin-12, Lyme disease, tick-borne

Introduction

Lyme borreliosis (LB) and human granulocytic ehrlichiosis (HGE) are tick-borne diseases caused by *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, respectively. LB is the most common tick-borne disease in Europe, with an annual incidence in Sweden of 69 cases per 100 000 inhabitants [1]. A typical debut symptom of LB is a circular rash called erythema migrans (EM). In the later stages LB can affect, for example, the nervous system, the joints and the skin. In some cases the disease develops into a chronic stage, with symptoms lasting longer than 6 months [2,3].

HGE is a newly discovered disease in humans. The first case was reported in the United States in 1994 [4] and in Europe in 1997 [5]. The symptoms are influenza-like, including fever, headache, myalgia and/or malaise. Laboratory findings may include leucopenia, thrombocytopenia and elevated liver enzymes [6]. Both LB and HGE can usually be treated with antibiotic therapy [6,7].

HGE has been observed to induce immunosuppression which can lead to opportunistic viral and fungal infections [8]. An experimental study showed that mice co-infected with *B. burgdorferi* and *A. phagocytophilum* had a decreased T helper type 1 (Th1) response, with diminished levels of interferon (IFN)-γ, interleukin (IL)-12 and tumour necrosis factor (TNF)-α compared to mice single-infected with *B. burgdorferi* [9]. The co-infected mice also developed more severe arthritis and showed an increased pathogenic load of both bacteria. Studies in animals indicate that *A. phagocytophilum* might have prolonged inhibitory effects on immune cells [10–12].

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An optimal eradication of infecting agents demands an early and powerful immune response of the correct type. The adaptive immune response is regulated by T lymphocytes that can be divided roughly into two mutually antagonistic subpopulations depending on their cytokine secretion patterns [13]. Th1 secrete, for example, IFN-γ and mediate activation of cytotoxic and phagocytic cells, including the production of opsonizing and complement-activating antibodies, whereas Th2 cells secrete, for example, IL-4, IL-5 and IL-13 which stimulates B cells to produce neutralizing antibodies and activates mast cells and eosinophils. Cytokines secreted from other cells, for example dendritic cells and macrophages, also play an important role for the deviation of Th-cells, where IL-12 induces Th1-type immunity. *Borrelia*-specific T cell responses in patients with LB have been reported by others and by us to be predominantly of Th1 type [14–18]. In addition, we have reported previously IgG subclass patterns reflecting Th1 responses in serum and cerebrospinal fluid from patients with neuroborreliosis (NB) [19] and an increase of the Th1-inducing cytokines IL-12 and IL-18 in cerebrospinal fluid from NB compared to controls [20]. Furthermore, we recently described increased TNF-α and IFN-γ secretion early in the infection in NB patients with a benign disease course compared with patients who developed chronic NB [21,22], suggesting strongly a beneficial role of Th1 responses early in human *Borrelia* infection. The importance of Th1 responses in eradication of *Borrelia* is supported by experimental studies, where mice that fail to eradicate the spirochetes and develop clinical signs of infection show weak Th1 responses initially in the infection, in contrast to resistant mice which show strong initial Th1 responses [23].

The immunological consequences of infection with *A. phagocytophilum* in humans have not yet been reported, to our knowledge. This prospective study was designed to identify patients with LB, with or without HGE, and to compare their cytokine secretion to non-exposed healthy controls. None of the patients included were found to be co-infected, but we did find evidence of patients exposed previously to *A. phagocytophilum* and currently infected with *B. burgdorferi*. Spontaneous as well as *Borrelia*-specific induced secretion of IFN-γ (Th1), IL-12 (Th1-inducing), IL-4, IL-5 and IL-13 (all Th2) was detected in peripheral blood with a sensitive enzyme-linked immunospot assay (ELISPOT)-technique.

Material and method

Patients

Thirty-eight patients were included in the study. Patients with EM ($n = 23$) were recruited between 2001 and 2003 from three health-care centres in the county of Östergötland. They all consulted a general practitioner for a suspected tick-borne disease. A questionnaire on symptoms associated commonly with LB and HGE was completed at the initial visit. Screening for antibodies to *A. phagocytophilum* in serum was performed by use of an indirect immunofluorescence assay, as described previously [24], in the acute and convalescent (after 6–8 weeks) stage. A general blood status analysis was also carried out. A diagnosis of LB required a confirmed or suspected tick bite within the last 30 days in combination with a clinically diagnosed EM with a diameter ≥ 5 cm [25]. All 23 patients received antibiotic treatment (penicillin V or doxycycline). Blood samples used for this study were drawn at the acute stage (0–22 days after initial visit to physician, median 9 days).

A non-exposed control group consisted of 15 healthy individuals, who were either staff at the hospital or blood donors. They were seronegative for *A. phagocytophilum* and *B. burgdorferi*.

Preparation of mononuclear cells

Peripheral blood mononuclear cells were separated from heparinized blood using gradient centrifugation on Lymphoprep (Medinor AB, Lidingö, Sweden), as described previously [17]. The cell concentration was adjusted to 1×10^6 lymphocytes/ml.

Elispot

The ELISPOT assay used was performed as described in detail elsewhere [16,17]. In short, the plates were coated with monoclonal antibodies, αIFN-γ, αIL-4, αIL-5, αIL-12 p70 and αIL-13 (Mabtech AB, Nacka, Sweden), diluted in sterile phosphate-buffered saline (PBS) to a final concentration of 15 μg/ml. The plates were incubated at 4°C overnight and then frozen at −20°C for no longer than 3 months.

The coated plates were thawed at 37°C, unspecific binding was blocked with cell tissue culture medium (TCM) [16] and 100 000 lymphocytes/well was added. To each well either TCM (to assess the spontaneous secretion) or an outer surface protein-enriched fraction of *Borrelia garinii* strain Ip90 (OF Ip90) [22] at a final concentration of 10 μg/ml was added in triplicate. Wells with TCM only were used as negative controls and as positive controls, cells were stimulated with the mitogen phytohaemagglutinin (PHA) at a final concentration of 20 μg/ml (Sigma-Aldrich, Stockholm, Sweden). The plates were then incubated at 37°C with 5% CO_2 and 95% humidity for 48 h.

Developing of spots, representing cytokine-secreting cells, was conducted with matched monoclonal antibodies, αIFN-γ, αIL-4, αIL-5, αIL-12 p70 and αIL-13, conjugated with biotin (Mabtech AB) diluted in PBS-Tween to a final concentration of 1 μg/ml, streptavidin conjugated with alkaline phosphatase (Mabtech AB) diluted 1 : 1000 in PBS-Tween and finally nitro blue tetrazolium (NPT) and bromochloroindolyl phosphate (BCIP) diluted in AP-buffer (AP conjugate substrate kit, Bio-Rad Laboratories AB, Sundbyberg, Sweden).

The spots were counted by the same person (S. J.) using an AID EliSpot Reader System version 2·6 (AID, Strassberg, Germany).

Data handling and statistics

The mean value of number of spots in the wells, spontaneous and *Borrelia*-stimulated, was calculated from the triplicates. The mean value of the spontaneous secretion was subtracted from the mean value of the *Borrelia*-stimulated secretion to obtain the specific antigen-induced secretion.

To compare the cytokine secretion, which was not normally distributed, between groups, the non-parametric Kruskal–Wallis test was used as a pretest and Dunn's multiple comparison test as a post-hoc test.

Student's *t*-test was used to compare lymphocyte and monocyte numbers between *Ap* seronegative LB and *Ap* seropositive LB patients. A *P*-value of $< 0{\cdot}05$ was considered significant.

The study was approved by the Research Ethics Committee of Linköping University.

Results

Patients

Of the 23 patients with EM, 15 were seronegative for *A. phagocytophilum* (termed *Ap* seronegative LB) and eight patients had antibodies to *A. phagocytophilum* (termed *Ap* seropositive LB) with titres $\geq 1:80$. However, *Ap* seropositive LB patients did not display any signs or symptoms of acute HGE, including elevated transaminase. In addition, their *A. phagocytophilum* antibody titres did not increase between the two samples drawn. Hence, HGE was considered not to be active.

The three groups (*Ap* seropositive LB, *Ap* seronegative LB and non-exposed healthy controls) did not differ with regard to sex or age, nor did the *Ap* seropositive LB and the *Ap* seronegative LB group differ in lymphocyte or monocyte counts in blood (Table 1). In both the *Ap* seropositive LB and the *Ap* seronegative LB groups one patient had rheumatoid arthritis and one had allergy, respectively. The patients were advised to consult their physician after their completed treatment if any new symptoms appeared. One of the *Ap* seropositive LB patients consulted a physician again 3 months after the initial visit. This patient had a new EM with the same location as the previous one and was diagnosed as having reactivation of borreliosis. The other 22 EM patients did not consult their physician after completed treatment, which was interpreted to mean that they had recovered.

Cytokine secretion

When *P*-values from the Kruskal–Wallis test indicated significant differences between the groups, further comparisons were performed with Dunn's multiple comparison test.

IL-12

When stimulated with *Borrelia* antigen, blood cells from the *Ap* seropositive LB patients showed a significantly lower number of Th1-inducing IL-12-secreting cells compared to *Ap* seronegative LB ($P < 0{\cdot}001$), (Fig. 1a). The non-exposed healthy controls also displayed a lower number of IL-12-secreting cells than the *Ap* seronegative LB ($P < 0{\cdot}01$), but no difference was noted between *Ap* seropositive LB and non-exposed controls. The Kruskal–Wallis test showed no significant differences in spontaneous IL-12 secretion between the groups (Fig. 2a).

IFN-γ

Ap seronegative LB displayed significantly ($P < 0{\cdot}05$) higher numbers of *Borrelia*-specific IFN-γ-secreting cells than non-exposed healthy controls (Fig. 1b). No difference was seen between *Ap* seropositive LB patients and the non-exposed healthy controls. The spontaneous secretion of IFN-γ was higher in the *Ap* seronegative LB group than in the non-exposed healthy control group ($P < 0{\cdot}05$) (Fig. 2b).

IL-4, IL-5 and IL-13

No significant difference was seen for IL-4 (Figs 1c and 2c), IL-5 (Figs 1d and 2d) or IL-13 (Figs 1e and 2e) when all

Table 1. Clinical and laboratory characteristics of patients (*Ap* seronegative LB = patients with erythema migrans seronegative for *Anaplasma phagocytophilum*; *Ap* seropositive LB = patients with erythema migrans seropositive for *A. phagocytophilum*; non-exposed healthy controls = individuals seronegative for *Borrelia burgdorferi* and *A. phagocytophilum*).

	Ap seronegative LB $n = 15$	*Ap* seropositive LB $n = 8$	Non-exposed healthy controls $n = 15$
Years of age, median (range)	50 (17–74)	50 (25–58)	47 (27–59)
Sex (F/M)	8/7	5/3	9/6
Blood lymphocyte count 10^9/l *†, mean (s.d.)	1·9 (0·3)	1·6 (0·5)	n.d.
Blood monocyte count 10^9/l *‡, mean (s.d.)	0·5 (0·1)	0·5 (0·1)	n.d.

**Ap* seronegative LB, $n = 13$; *Ap* seropositive LB, $n = 5$. †Normal value, 1·1–4·8 10^9/l. ‡Normal value, 0·1–1·0 10^9/l. *Ap*, *Anaplasma phagocytophilum*; LB, Lyme borreliosis; F, female; M, male; n.d., not done.

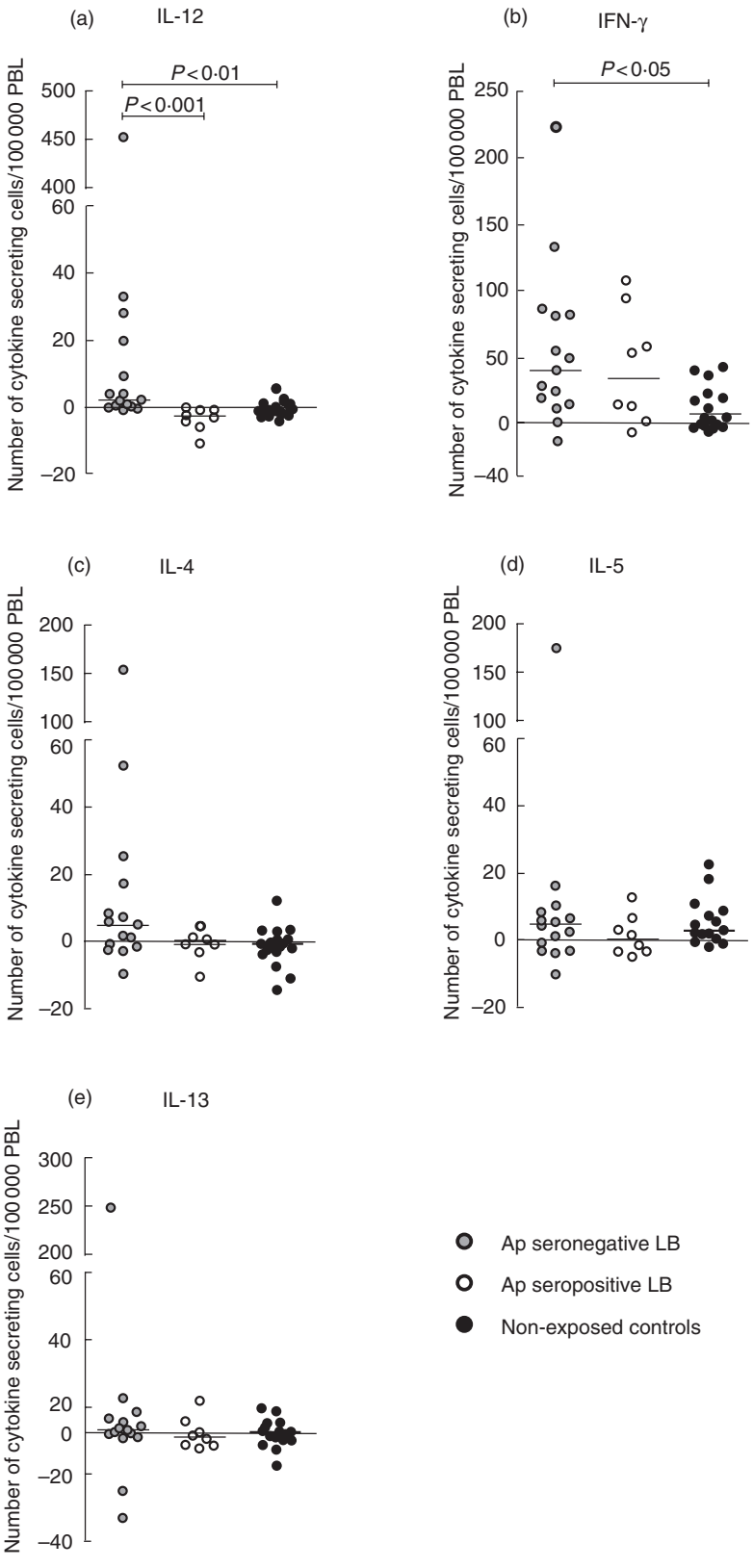

Fig. 1. Number of *Borrelia*-specific cytokine secreting cells/100 000 peripheral blood lymphocytes (PBL) detected by enzyme-linked immunospot. Values are net values, thus the number of spontaneously cytokine-secreting cells has been subtracted. *P*-values show statistically significant differences from comparison with Dunn's multiple comparison test that was carried out when the Kruskall–Wallis test showed significant differences in comparisons of all groups. Each point represents one individual and the lines mark the median values. *Ap* seronegative LB, $n = 15$ (patients with erythema migrans seronegative for *Anaplasma phagocytophilum*), *Ap* seropositive LB, $n = 8$ (patients with erythema migrans seropositive for *A. phagocytophilum*), non-exposed healthy controls $n = 15$ (individuals seronegative for *Borrelia burgdorferi* and *A. phagocytophilum*). Note the different scales in the diagrams.

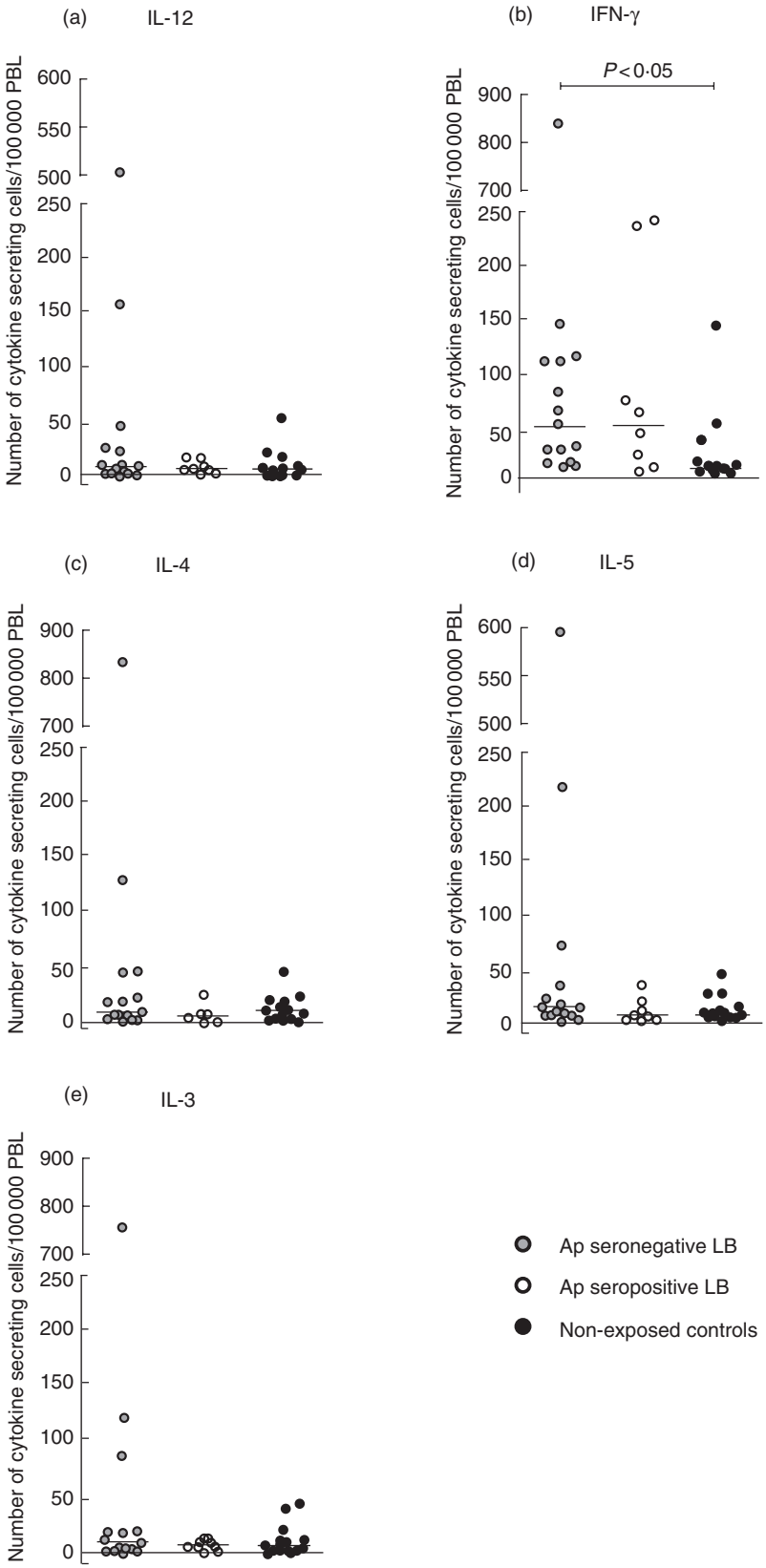

Fig. 2. Number of spontaneously cytokine-secreting cells/100 000 peripheral blood lymphocytes (PBL) detected by enzyme-linked immunospot. *P*-values show statistically significant differences from comparison with Dunn's multiple comparison test that was carried out when the Kruskall–Wallis test showed significant differences in comparisons of all groups. Each point represents one individual and the lines mark the median values. *Ap* seronegative LB, $n = 15$ (patients with erythema migrans seronegative for *Anaplasma phagocytophilum*), *Ap* seropositive LB, $n = 8$ (patients with erythema migrans seropositive for *A. phagocytophilum*), non-exposed healthy controls $n = 15$ (individuals seronegative for *Borrelia burgdorferi* and *A. phagocytophilum*). Note the different scales in the diagrams.

groups were compared with the Kruskal–Wallis test, and therefore no further comparisons were carried out.

Discussion

The agent of HGE, *A. phagocytophilum*, and the agent of LB, *B. burgdorferi*, are transferred by the same species of ticks, *Ixodes ricinus* [26,27]. Although treatable with antibiotics, there are findings suggesting that *A. phagocytophilum* might remain hidden in different tissues. Chang *et al.* found *A. phagocytophilum* DNA in muscle, adrenal gland and peritoneum, but not in blood from afebrile horses [28]. Bacterial DNA was, however, detected in blood during and around the febrile period. *A. phagocytophilum* have also been detected after infection in various human organs, for instance spleen, lung, heart and kidney [29].

We show in this study that patients exposed previously to *A. phagocytophilum* and currently infected with *B. burgdorferi* (termed *Ap* seropositive LB) have a lower number of *Borrelia*-specific cells secreting IL-12 compared to *Ap* seronegative LB patients. IL-12 is important at the onset of an immune response, as it stimulates natural killer (NK) cells and induces Th1 responses and thereby enhances the cytotoxic and phagocytic abilities of the immune system. When challenged by the *Borrelia* spirochete the immune defence is shaped towards a strong Th1 response with high levels of IFN-γ [17,19,30]. In addition, we have recently found indications for the activation of cytotoxic responses in *Borrelia* infection, supporting further the significance of Th1 responses in LB [31]. A weak or delayed Th1 response to *Borrelia* would probably cause a prolonged infection, including a risk for the development of chronic LB [22].

The lack of *Borrelia*-specific IL-12 secretion in *Ap* seropositive LB patients might suggest a reduced Th1 response. However, there were no differences between the *Ap* seronegative LB and *Ap* seropositive LB in the *Borrelia*-specific IFN-γ production. On the other hand, the *Ap* seronegative LB had a significantly higher number of cells secreting IFN-γ in response to *Borrelia* than the non-exposed healthy controls, but this difference was not seen between the *Ap* seropositive LB and the controls, indirectly indicating decreased IFN-γ response in the *Ap* seropositive LB group.

Our findings are in line with animal studies, where a reduction in lymphocyte proliferation, in response to mitogen stimulation, has been shown for up to 6 weeks after infection with *A. phagocytophilum* [11,12]. Furthermore, serum from previously *A. phagocytophilum*-infected sheep inhibited proliferation of cells from non-infected individuals [11,32]. Whist *et al.* showed a suppressed *Mycobacterium avium*-specific IFN-γ response seen up to 31 days post-inoculation [10]. Based on these studies, and studies indicating that *A. phagocytophilum* might remain hidden in different tissues [28,29], we suggest that our results could possibly imply a general immunosuppression induced by *A. phagocytophilum*, which may be long-standing even after the infection is resolved. This concept must, however, be confirmed in further studies.

The *Borrelia* antigen used in the present study has been shown previously to discriminate between *Borrelia* infections and seronegative controls regarding secretion of IFN-γ and IL-4 [18,22]. We chose the ELISPOT method because of its high sensitivity. There are, however, disadvantages with this method, one being the missed information of the amount of cytokine secreted, as ELISPOT detects only the number of cells secreting a specific substance. Spontaneously secreted IL-4 [33] and IL-12 are found usually in very low concentrations which are not possible to detect using, for example, enzyme-linked immunosorbent assay, whereas ELISPOT can detect even a single cytokine-secreting cell. The functional relevance of such small amounts of cytokine is uncertain. However, IL-4 and IL-12 are regarded as potent cytokines, therefore low levels might have significant effects.

This study was not designed to follow-up the patients clinically for a long period, because adequate antibiotic treatment of EM is believed to lead to full recovery in all patients [7], as was also the case in this study. Due to the relatively small number of patients, confounding factors such as allergies or autoimmune diseases, which may affect the immunological response, was not analysed.

In conclusion, this study indicates that patients exposed previously to *A. phagocytophilum* and currently infected with *B. burgdorferi* have a lower *Borrelia*-specific secretion of IL-12 compared to *A. phagocytophilum* seronegative patients infected with *B. burgdorferi*. We suggest that this could mirror a reduced Th1 response caused by *A. phagocytophilum*. Whether this leads to impaired eradication of *B. burgdorferi*, or even has other implications in human immune homeostasis, remains to be established.

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